IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Takumi TERATANI et al. : Group Art Unit: 1633
Serial No. 10/591,407 : Examiner: Quang NGUYEN
Filed: December 8, 2006 :
For: RAT EMBRYONIC STEM

DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, Takahiro Ochiya, declare:
That I am a citizen of Japan, and my post office address is National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045 Japan;
That my education and employment history is as shown in my Curriculum Vitae attached hereto (Attachment 1):
That I am a co-inventor of the above-identified U.S. patent application SN 10/591,407 and directed and supervised the following experiment, which was carried out to demonstrate that rat embryonic stem (ES) cells defined in the instant specification cannot be established by the method described in the Examples of Loring (WO 99/27076) (EXPERIMENT 1) and that alkaline phosphatase-positive rat ES cells can be established by the method of claim 8, even when the culture mediums used contain 2% serum (EXPERIMENT 2), the results of which follow hereunder;

**[EXPERIMENT 1]**

Reproducibility test of rat ES cell establishment by the method of Loring (WO 99/27076)

[Materials & Methods]

*Culture media*

1) Medium for ES cell establishment

DMEM High Glucose medium (MP Bio)

2 mM L-Glutamine (MP Bio)

20% Fetal bovine serum (FBS) (Millipore)

1% Non Essential Amino Acid Stock (MP Bio)

1% Antibiotic antimycotic (GIBCO)

0.1 mM 2-Mercaptoethanol(GIBCO)

2000 U/mL mouse leukemia inhibitory factor (mLIF) (Millipore)

2) Media for maintaining ES cells

i) Maintenance medium A

DMEM High Glucose medium (MP Bio)

2 mM L-Glutamine (MP Bio)

15% FBS (Millipore)

1% Non Essential Amino Acid Stock (MP Bio)

1% Antibiotic antimycotic (GIBCO)

0.1 mM 2-Mercaptoethanol(GIBCO)

ii)Maintenance medium B

DMEM High Glucose medium (MP Bio)

2 mM L-Glutamine (MP Bio)

15% FBS (Millipore)

1% Non Essential Amino Acid Stock (MP Bio)

1% Antibiotic antimycotic (GIBCO)

0.1 mM 2-Mercaptoethanol(GIBCO)

20 ng/mL basic fibroblast growth factor (bFGF) (AbD Serotec)

3) HAT-containing media for maintaining ES cells

i) HAT(+) maintenance medium A

Maintenance medium A

2% HAT Supplement Solution (MP Bio)

ii)HAT(+) maintenance medium B

Maintenance medium B

2% HAT Supplement Solution (MP Bio)

*Feeder cells*

Mitomycin C-treated neomycin-resistant mouse embryonic fibroblasts (MEF; Millipore) were used as feeder cells, which were maintained in a medium having the following composition:

DMEM (GIBCO)

10% FBS (EQUITECH-BIO, Lot No. SFB30-1502)

1% 1×Antibiotic antimycotic (GIBCO)

*Mouse ES cells*

HPRT-deficient mouse ES cell line ES-E14TG2a was obtained from American Type Culture Collection (ATCC No. CRL-1821).

[Results]

(1) *Determination of culture conditions of mouse ES cells*

To exclude the possibility of contamination of mouse ES cells co-cultured with rat cells, we determined culture conditions that no mouse ES cells survived after the cultivation.

ES-E14TG2a cells were cultured on feeder cells in either of HAT(+) maintenance medium A and HAT(+) maintenance medium B for 3 days. The results are shown in Figure 1. As shown in Figure 1(A) and (B), mouse ES cells died whereas feeder cells survived, when they were co-cultured in the HAT-containing media for 3 days. On the other hand, when they co-cultured in a medium for maintaining ES cells without HAT, colonies of mouse ES cells were observed as shown Figure 1(C).

Therefore, we determined to culture candidates of rat ES cells obtained by co-culture with the mouse ES cells in HAT(+) maintenance medium A or B for 3 days prior to confirmation of the presence of rat ES cell colonies.

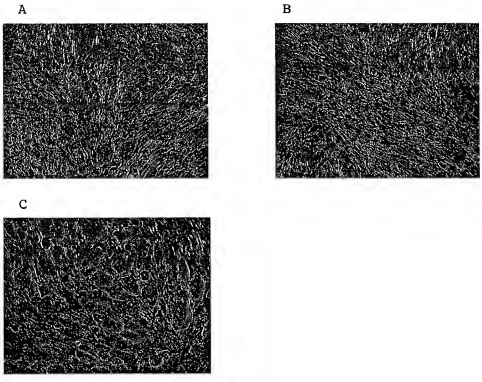



Figure 1. Micrographs of mouse ES cells (ES-E14TG2a) cultured on feeder cells in the presence (A, B) and absence (C) of HAT. The mouse ES cells and feeder cells were co-cultured for 3 days in HAT(+) maintenance medium A (A), HAT(+) maintenance medium B (B) and a medium for maintaining ES cells without HAT (C). No mouse ES cell colony was observed due to the death of mouse ES cells, when culturing in HAT-containing media (A, B).

(2) *Establishment of rat ES cells*

We examined whether or not rat ES cells can be established by co-culturing inner cell mass (ICM) cells obtained from rat blastocysts with mouse ES cells in the same manner as Example 5 of Loring (WO 99/27076).

Uteri of E4.5 to E5 pregnant Wistar rats were perfused with the above-identified Medium for ES cell establishment to give rat blastocysts. Zona pellucida were removed with Tyrode's solution (Ark Resource), and the blastocysts were transferred onto mitomycin C-treated feeder cells in 6-well plates and cultured with the medium for ES cell establishment containing 20% FBS and mLIF. Three days

after, rat ICM cells derived from the blastocysts were excised using Stem Cell Cutting Tool (Vitrolife), incubated in 1 mM EDTA for 30 minutes and subjected to the culture for inducing rat ES cells.

Dispersed ICM cells were sown onto mitomycin C-treated feeder cells in each well of 6-well plates, and mouse ES cells (ES-E14TG2a; $1.5 \times 10^5$ cells/well) were added to the each well. These cells were co-cultured in Maintenance medium A (condition 1) or Maintenance medium B (condition 2).

Three days after, ES cell colonies in the wells were treated with 0.25% trypsin/1 mM EDTA for 15 minutes, the dissociated cells were diluted 1:10, transferred onto mitomycin C-treated feeder cells in 6-well plates. The cells were subcultured every 3-5 days for 4 times in the same manner, and then the resulting ES cell colonies were dissociated in the same manner as used in subculture, the dissociated cells were transferred onto mitomycin C-treated feeder cells in 6-well plates without dilution and cultured in the same Maintenance medium except containing HAT (i.e., condition 1: HAT(+) maintenance medium A; condition 2: HAT(+) maintenance medium B) for 3 days, while exchanging the culture medium everyday, to kill the mouse ES cells.

The overall culture protocols are summarized in Figure 2.

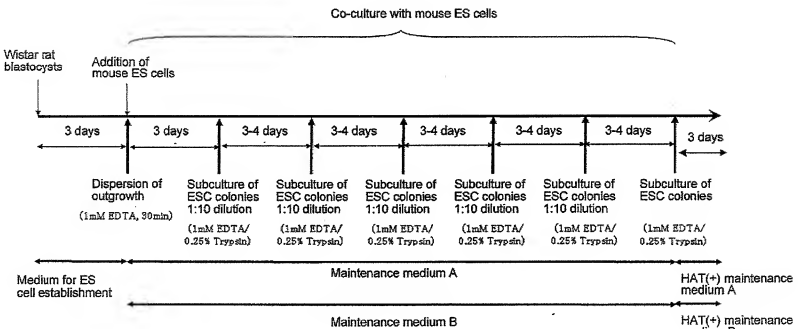
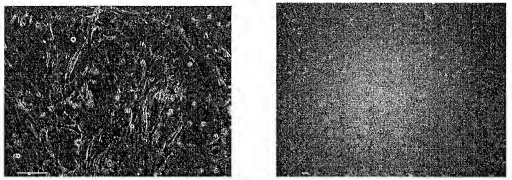



Figure 2. Culture protocols of rat ES cell establishment

(3) Confirmation of establishment of rat ES cells

After the culture in HAT(+) maintenance medium A or B for 3 days for killing the mouse ES cells in (2) above, the presence or absence of ES cell colonies in the wells were observed by microscopy. The results are shown in Figure 3. Under both condition 1 and condition 2, no ES cell colony was observed in any well, demonstrating that rat ES cells cannot be established.

A



B

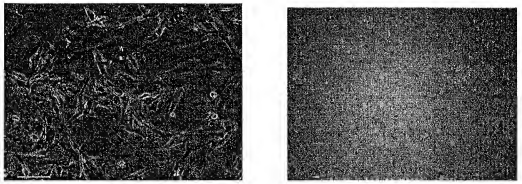


Figure 3. Micrographs of co-culture of candidates of rat ES cells and mouse ES cells on feeder cells in HAT-containing media. The cells were cultured in HAT(+) maintenance medium A (A) or HAT(+) maintenance medium B (B) for 3 days. Under both conditions, no ES cell colony was observed.

[Conclusion]

It was confirmed that rat ES cells cannot be established by the method described in Example 5 of Loring (WO

99/27076).

**[EXPERIMENT 2]**

Establishment of rat ES cells using culture media containing 2% FBS

[Materials & Methods]

*Culture media*

1) Medium for ES cell establishment
   DMEM/F12 1:1 Mixture medium (GIBCO)
   2 mM L-Glutamine (MP Bio)
   2% Fetal bovine serum (FBS) (Millipore)
   1% Non Essential Amino Acid Stock (MP Bio)
   1% Antibiotic antimycotic (GIBCO)
   1 mM Sodium pyruvate(MP Bio)
   0.1 mM 2-Mercaptoethanol(GIBCO)
   1% Nucleoside (Millipore)
   10% Knockout Serum Replacement (GIBCO)

2) Media for maintaining ES cells
   DMEM/F12 1:1 Mixture medium (GIBCO)
   2 mM L-Glutamine (MP Bio)
   2% Fetal bovine serum (FBS) (Millipore)
   1% Non Essential Amino Acid Stock (MP Bio)
   1% Antibiotic antimycotic (GIBCO)
   1 mM Sodium pyruvate(MP Bio)
   0.1 mM 2-Mercaptoethanol(GIBCO)
   1% Nucleoside (Millipore)
   10% Knockout Serum Replacement (GIBCO)
   1000 U/mL rat leukemia inhibitory factor (rLIF) (Millipore)

*Feeder cells*

Mitomycin C-treated neomycin-resistant mouse embryonic fibroblasts (MEF; Millipore) were used as feeder cells, which were maintained in a medium having the following composition:

DMEM (GIBCO)
10% FBS (EQUITECH-BIO, Lot No. SFB30-1502)
1% 1×Antibiotic antimycotic (GIBCO)

[Results]

(1) *Establishment of rat ES cells*

We examined whether or not alkaline phosphatase-positive rat ES cells can be established in the same manner as the method described in Example 1 of the instant specification, even when using culture media containing 2% FBS in place of serum-free media.

Uteri of E4.5 to E5 pregnant Wistar rats were perfused with the above-identified Medium for ES cell establishment to give rat blastocysts. Zona pellucida were removed with Tyrode's solution (Ark Resource), and the blastocysts were transferred onto mitomycin C-treated feeder cells in 6-well plates and cultured with the medium for ES cell establishment containing 2% FBS. Seven days after, the outgrowth of blastocysts was mechanically dissociated using Stem Cell Cutting Tool (Vitrolife). The dissociated cells were transferred onto mitomycin C-treated feeder cells in 6-well plates, cultured in the Medium for maintaining ES cells containing 2% FBS for 5 days. The emerged ES cell colonies were dispersed using Stem Cell Cutting Tool (Vitrolife), the dispersed outgrowth was transferred onto mitomycin C-treated feeder cells in 6-well plates, and cultured in the Medium for maintaining ES cells containing 2% FBS for 4 days. The emerged ES cell colonies were dispersed using Stem Cell Cutting Tool (Vitrolife), the dispersed ES cell colonies were transferred onto mitomycin C-treated feeder cells in 6-well plates, and cultured in the Medium for maintaining ES cells containing 2% FBS to establish rat ES cells. The overall culture protocol is summarized in Figure 4.

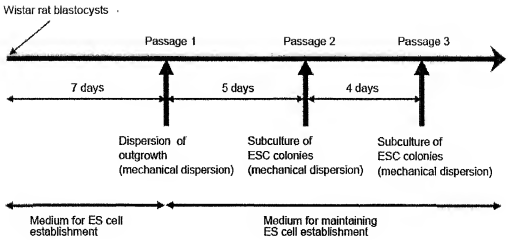



Figure 4. Culture protocol of rat ES cell establishment

(2) *Alkaline phosphatase assay*

The alkaline phosphatase activities of the rat ES cells established in (2) above (passage 3) were assayed by staining alkaline phosphatase-positive cells using Vector Blue Alkaline Phosphatase Substrate (Vector Labs). As a result, the rat ES cells established were shown to be alkaline phosphatase-positive (Figure 5). Figure 6 shows a phase-contrast micrograph of the alkaline phosphatase-positive rat ES cell colony A observed in Figure 5.

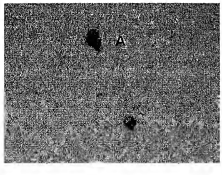



Figure 5. Staining assay of alkaline phosphatase-positive rat ES cell colonies.

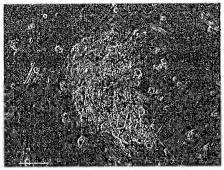


Figure 6. phase-contrast micrograph of the alkaline phosphatase-positive rat ES cell colony A observed in Figure 5.

[Conclusion]

We confirmed that alkaline phosphatase-positive rat ES cells can be established by the method of claim 8 of the instant application, wherein the serum concentration of the culture media is 2%. This demonstrates that the unexpected result (i.e., successful establishment of rat ES cells) commensurate in scope with the claimed invention.

That I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 18 day of March, 2011.

Takahiro OCHIYA

## Curriculum Vitae

**Takahiro OCHIYA**

| | |
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| **Title:** | Chief, Division of Molecular and Cellular Medicine, Translational Research Group, National Cancer Center Research Institute |

**Educational History:**

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|---|---|
| 1988 | Osaka University, Graduate School of Medicine (Ph.D. awarded) |

**Professional History:**

| | |
|---|---|
| 1988-1992 | Research Assistant, Institute for Molecular and Cellular Biology, Osaka University |
| 1993-1998 | Section Head, Genetics Division, National Cancer Center Research Institute |
| 1998-2010 | Section Head, Section for Studies on Metastasis, National Cancer Center Research Institute |
| 1991-1992 | The Barnham Institute Medical Research, La Jolla, CA USA |
| 2004- | Visiting Professor, Waseda University, Graduate School of Science and Engineering, Bioscience and Biomedical Engineering |
| 2008- | Visiting Professor, Tokyo Institute of Technology School and Graduate School of Bioscience and Biotechnology |
| 2010- | Chief, Division of Molecular and Cellular Medicine, Translational Research Group, National Cancer Center Research Institute |

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|---|---|
| **Society:** | Japan Society of Gene Therapy<br>Japanese Cancer Association<br>Japanese Biochemical Society<br>The Molecular biology Society of Japan |

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